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(54) Title: WHOLE CELL ASSAY			
(57) Abstract			
<p>This invention provides a method for determining the presence of specific cell types in a mixed population of cells. This method involves the identification and detection of markers for the target cell types, the preparation of primers specific for the markers, and the use of reverse transcriptase reaction technology to amplify and detect these markers. The method of this invention is sensitive enough to detect the presence of as few as 100 target cells in a cell population. Specific examples of target cells include human chondrocyte cells and human keratinocyte cells.</p>			

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WHOLE CELL ASSAY

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is related to and claims the benefit of provisional application serial number 60/022,636, filed July 26, 1996, and provisional application serial number 60/035,415, filed January 23, 1997.

BACKGROUND OF THE INVENTION

This invention relates to a rapid and sensitive assay and method, for confirming the presence of specific mRNA species which are present in a cell suspension. The assay and method of this invention utilizes whole cells as a starting material for a reverse transcriptase polymerase chain reaction ("RT-PCR") analysis. Primers are designed and used to amplify specific cDNA sequences conforming to the mRNA. Specific and positive identification of cell types present in a mixed population of cells can thus be confirmed with only minimal time and sample manipulation.

To determine cell type with currently available technologies, it is possible to use a variety of strategies. One such strategy is to determine cell type by examining the cell or tissue morphology. The disadvantage of this method is that it requires an extensive amount of training, and even then it is highly subjective and not consistently reproducible. This can be improved upon by applying histological stains to the cells or tissues. In general, histology is time consuming, and the stains are not very specific. In addition, the staining and fixing of cells or tissues often involves hazardous chemicals and facilities for working with and disposing of these materials.

A second strategy for determining cell type is to look for protein markers known to be expressed only by specific cell types. Examples would be immunohistology or FACS technology. Although this is much more specific than examining morphology, these methods rely heavily on the availability of antibodies raised against the protein marker of interest. However, antibodies are often not readily available. In addition, every antibody-antigen interaction is unique, and requires conditions to be optimized on an individual basis.

A third strategy for cell typing is to examine the mRNA that is transcribed by cells. While every cell type has a unique mRNA profile, the methods employed for working with RNA are independent of the specific mRNA being examined. Techniques are applicable to a broad range of cell sources with at most very minor modifications. Northern blotting and ribonuclease protection assays are two methods that can detect and quantify the presence of a specific mRNA. However, both of these methods require substantial amounts (1 to 20 micrograms) of RNA which generally needs to be purified first. *In Situ* hybridization and *in situ* PCR can be done using much less starting material, but both of these methods are technically difficult and lengthy to perform and difficult to analyze.

In the past few years, the study of gene expression in eukaryotic cells has benefited from several technical improvements in the sensitivity, specificity, and time requirements for the detection of target mRNA. The RNase protection assay is an improvement over northern blot analysis since it can be quantitative, requires very little starting material, and can be performed directly on whole cells without prior RNA purification (Strauss and Jacobowitz, Brain Res. Mol. Brain Res., 20, pages 229-239, (1993). However, RNase protection assays still require substantial amounts of time and manipulation. The RT-PCR assay allows for the detection of rare mRNA transcripts from very little RNA, Klebe et al., BioTechniques, 21 (6), pages 1094-1100 (1996). However,

several steps are still required, including RNA isolation and first strand cDNA synthesis, followed by PCR amplification. These limitations make it difficult to use either the RNase protection assay or the RT-PCR assay as a rapid assay for screening cells for a specific target mRNA.

Accordingly, it is an objective of this invention to provide a simplified procedure for overcoming the shortcomings of the prior art by allowing specific and positive identification of a target mRNA within a cell culture in a rapid fashion and with minimal sample manipulation.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is an electrophoresis photograph showing the presence of aggrecan and GAPDH in passaged human chondrocytes and human dermal fibroblasts.

Figure 2a is a photograph of an agarose gel showing the results of an assay performed on total RNA isolated from chondrocytes and fibroblasts.

Figure 2b is a photograph of a Southern blot showing the results of an assay performed on total RNA isolated from chondrocytes and fibroblasts.

Figure 3 is a schematic diagram of genomic and cDNA templates with primer locations as indicated. Intervening sequences are shown in the genomic DNA but are absent from the cDNA and mRNA.

Figure 4 is a photograph of an agarose gel showing the results of an assay performed on total RNA isolated from chondrocytes and fibroblasts with and without RNase.

Figure 5 is a photograph of an agarose gel showing the results of an assay performed on total RNA isolated from chondrocytes and fibroblasts with and without RNase and DNase.

Figure 6 is a photograph of a Southern blot showing the presence of GAPDH in human chondrocytes and fibroblasts from genomic DNA template (250 bp) and mRNA template (146bp).

Figure 7 is a photograph of an agarose gel comparing the presence of RNase A and RNase inhibitor on the detection of aggrecan and GAPDH from intact human chondrocytes and fibroblasts.

Figure 8 is a photograph of an agarose gel showing the presence of aggrecan and GAPDH from intact human chondrocytes and fibroblasts.

Figure 9 is a photograph of a Southern blot showing the presence of aggrecan and GAPDH from intact human chondrocytes and fibroblasts.

Figure 10 is a photograph of an agarose gel comparing the presence of RNase A and RNase inhibitor on the detection of aggrecan and GAPDH from intact human chondrocytes and fibroblasts.

SUMMARY OF THE INVENTION

This invention is directed to a rapid and accurate method for the determination of a specific type of cell, such as a chondrocyte cell, in a mixed population of cells. The method of this invention uses whole cells as starting materials. Markers specific for the target cells are identified, and primers for the markers are constructed. Preferably, the primers are constructed to yield a nucleic acid product from reverse-transcribed RNA template which is smaller than DNA amplified from the corresponding genomic template.

In carrying out the method of this invention, the cells are separated from the remaining components of a liquid sample by any convenient means, such as centrifugation. Preferably, the cells are formed into pellets. A reaction mixture is formed by combining the pellets, RT-PCR medium, a cell lysing agent or detergent, such as Tween-20, and optionally, an RNase inhibitor to minimize degradation of the mRNA. The reaction is maintained at a sufficiently high temperature, i.e. from about 60° C to about 90° C, in order to reduce mRNA

degradation by temporarily inactivating Rnases and to reduce false priming.

The method of this invention is particularly useful for analyzing samples of human cartilage tissue which are surgically removed from a patient as an adjunct to a cartilage repair procedure. After removal from the patient, the sample is analyzed for the presence of aggrecan mRNA which is specific to chondrocyte cells. If sufficient cells are present in the sample, as determined by the procedure of this invention, the cells may be cultured in order to form a patch to repair a cartilage defect. It will be readily appreciated that a similar approach could be used to replace keratinocytes.

DETAILED DESCRIPTION OF THE INVENTION

The method of this invention utilizes molecular markers that are known to be specific for a certain cell type. Preferably, a modified single-tube RT-PCR analysis is performed to amplify the signal for these markers. Such techniques are well known in the art, and are available commercially. It will be understood that any amplification technique known in the art may be used as long as it achieves the objectives of the instant method and assay.

The method and assay can be used in any procedure where it is necessary to confirm the presence of specific cell types in a mixed population of cells. More generally, it can be used wherever a specific RNA is expected to be transcribed by some or all cells. A few examples are chondrocytes (aggrecan mRNA), keratinocytes (keratin mRNA), viral or bacterial RNA, cells expressing an mRNA isoform with a deletion or insertion mutation, and RNA transcribed only in cancers.

The ability to use whole cells as a starting material, without first having to purify mRNA or total RNA from the cells, greatly reduces the amount of time and sample handling required for analysis of gene expression. The entire procedure can be carried out in a

single tube with a single buffer and virtually no manipulation of the samples. In many situations, currently available methods of cell-typing are inadequate because they require too much time or manipulation, or because they are too subjective.

Primers with fairly high melting temperatures are chosen to amplify a short section of mRNA where an intron is present in the corresponding genomic sequence. Such primers allow a distinction to be made between signal generated from a genomic DNA template, which is present in all cells, and the reverse-transcribed mRNA, which is cell-type specific. A high primer annealing temperature allows for very specific interactions between primers and the template to occur. It also enables the use of a two-step PCR amplification in which the annealing and extension steps are combined. The short distance between primers, combined with the high processivity of the enzyme and the two-step PCR amplification, greatly reduces the cycling times required to generate a signal from specific mRNAs.

The quickness and simplicity of this assay is optimized by having an enzyme, or a mix of enzymes, with thermostable reverse transcriptase activity as well as DNA polymerase activity in a single buffer. This dual activity/single buffer capacity enables the assay to be completed in a single tube that requires no manipulation during the assay. The thermostable polymerase rTth is a thermostable enzyme with both reverse transcriptase and DNA polymerase activities, and the buffer system, such as the PCR reaction buffer available from Perkin Elmer Cetus, permits both activities.

The assay of this invention can be used for determining whether a specific gene is transcribed in a given cell population, by choosing primer pairs that distinguish RNA signal from genomic DNA signal. The method utilized herein is able to generate a signal directly from whole cells, and proves to be a specific, simple, rapid and sensitive assay that requires minimal handling time and manipulation. This method can be used in order to

confirm the presence of a specific target mRNA in a mixed population of cells.

The method of this invention is highly sensitive in detecting specific, low abundant mRNAs from less than about 1000 cells, and as few as 100 cells. Furthermore, the amplified DNA fragments are generated from transcribed genes, not from genomic DNA. This procedure simplifies qualitative gene expression analysis significantly, as whole cells are used as a template in a single tube, single buffer, one-enzyme RT-PCR reaction.

Accurate quantitation of RNA using RT-PCR has been made possible by recent innovations that reduce the introduction of artifacts due to minor variations in conditions between samples during the reverse transcription and amplification steps.

An assay that allows high throughput and quantitative screening of cells for specific target mRNAs would have many potential applications. For example, conditions could be assayed that promote transcription of specific mRNAs by cells *in vitro*, heterogenous cell populations could be tested for the abundance of a specific cell type, or transcription levels from plasmid transfection could be measured and optimized.

The various features and aspects of this invention are illustrated in the following examples, which should not be considered as limitations the scope of the invention as defined in the appended claims.

EXAMPLE 1

Primers were designed to amplify a 280bp region of the G3 domain of aggrecan mRNA, which is a chondrocyte specific marker. The primers were chosen so that they would not amplify sequence from genomic DNA template, since the region between the primers is predicted to span an intron too large to be amplified under the reaction conditions. Primers were also chosen as a positive controls for amplification. The primers were designed to

co-amplify a 146bp region of GAPDH, which is a housekeeping gene transcribed in all cell types.

Passaged human chondrocytes grown in monolayer on tissue culture treated plastic are released from the plastic with trypsin, which is inactivated by the addition of five volumes of cell media that contains 10% Fetal Bovine Serum (FBS). A small aliquot of the cell suspension is removed for counting on a hemocytometer while the cells are pelleted in a clinical centrifuge. The cell pellet is resuspended in 10ml of phosphate buffered saline (PBS), and 10,000 cells are pipetted into a 0.2ml thin-walled PCR tube. Cells are pelleted in the PCR tube by centrifugation, and the PBS supernatant is removed. RT-PCR reaction is performed using techniques optimized to reduce mRNA degradation as described below.

An RT-PCR reaction mix, briefly preheated to 90° C in a thermocycler, is added directly to the cell pellet. This mixture consists of:

	Tween-20	0.125µl	0.5% (V/V)
	Placental RNase Inhibitor		1.25µl
	500U/ml		
each	Primers	4µl	11.25 pmol
	dNTPōs	3µl	300µM each
	5x buffer	5µl	1/5 volume
	Mn(OAc) ₂	2.5µl	2.5mM
	Water	to 24µl	

The reaction mix is placed into the thermocycler set to 90° C. Enzyme rTth (1µl = 2.5U) is added to the reaction, which is then quickly vortexed and immediately placed back into the thermocycler. The program then continues as follows:

63.5° C (reverse primer's optimal annealing temperature) for 15 minutes
 92° C for one minute
 35° cycles of:
 92° C for 10 seconds
 63.5° C (reverse primer's optimal annealing temperature) for 20 seconds
 72° for three minutes

Hold at 4° C until ready to analyze.

The specificity of the assay was established using human fibroblast cells as a template for RT-PCR. The 280bp band corresponding to aggrecan was amplified only from chondrocytes or purified chondrocyte RNA, and not from fibroblasts or purified fibroblast RNA. A similar assay run using purified RNA showed that there is no difference in the size of the amplified fragments from whole cell template versus isolated RNA template.

The amplified products are analyzed by agarose gel electrophoresis, and the results are shown in Figure 1.

EXAMPLES 2-9

The following general procedure applies to Examples 2-9.

Passaged human articular chondrocytes and dermal fibroblasts were grown in monolayer on tissue culture plastic and fed DMEM with 10% fetal bovine serum. Cells were released from the tissue culture treated plastic with trypsin/EDTA, which was then inactivated by addition of five volumes of media that contains 10% FBS. A small aliquot of the cell suspension was removed for counting on a hemacytometer while the cells were pelleted in a clinical centrifuge. The cell pellet was resuspended in PBS, and 10,000 cells were pipetted into a 0.2 ml thin-walled PCR tube. Cells were pelleted in the PCR tube by centrifugation at 3000 rpm in a table-top microcentrifuge, and then the PBS supernatant was removed.

Primers were designed using the Oligo 5.0 program (National Biosciences Inc., Plymouth MN). Primers have a predicted melting temperature of greater than 85° C, and a predicted optimal annealing temperature between 63° C and 64° C. Each primer was chosen to anneal completely within a single exon of mRNA sequence, and the region between the forward and reverse primer was predicted to span an intron. With this primer design, the sequence amplified

from reverse transcribed RNA template yields a smaller product compared to the sequence amplified from genomic DNA template. The amplified product, including primers, was between 100 and 500 base pairs, which allowed for short cycle times.

Primer sequences, 5' to 3', are:

Aggrecan sense: CCAGGAGGTATGTGAGGAGGGCTGGAACAAG

(SEQ ID NO:1)

Aggrecan antisense: AGTTGTCAGGCTGGTTGGGGCGCCAGTTCT

(SEQ ID NO:2)

GAPDH sense: GCACCAGGTGGTCTCCTCTGACTTCAACAGCGA

(SEQ ID NO:3)

GAPDH antisense: TCCACCACCCTGTTGCTGTAGCCAAATTCGTT

(SEQ ID NO:4)

Primers were made and purified by reverse phase cartridge by Genosys Biotechnologies (Woodlands, TX).

An RT-PCR reaction was performed using *rTth* enzyme and buffer conditions optimized by Perkin Elmer Cetus Corporation (EZ *rTth* RNA PCR Kit, Roche Molecular Systems Inc., Branchburg NJ) to allow both reverse transcriptase and DNA polymerase activity. Reaction volume was 25 μ l, consisting of 0.5% (v/v) Tween 20, 11.25 pmol each primer, 300 μ M each dNTP, 1x reaction buffer, 2.5 mM Manganese Acetate, and water. Indicated samples had either 500 U/ml Placental RNase Inhibitor (Gibco, Grand Island NY) or RNase A/T₁ (20U/ml RNase A and 800U/ml RNase T₁), or 80U/ml DNase I (Ambion, Austin TX). A master mix was prepared and heated to 90° C in a heat block, then 24 μ l was dispensed into PCR tubes that contain 10,000 cells. The reactions were placed into an MJ-Research PTC-100 thermocycler set to 90° C. One microliter (2.5 units) of enzyme *rTth* was added to the reactions, which were then quickly vortexed and immediately placed back into the thermocycler. A 5 minute reverse-transcription step was performed at 63.5° C followed by a 60 second

denaturation step at 92° C. The cDNA was then amplified by 35 cycles of 10 second 92° C denaturation steps and 20 second annealing/extension steps at 63.5° C. A final extension step of 3 minutes at 72° C completed the thermocycler program, and the reactions are held at 4°C until ready for agarose gel electrophoresis. For some samples, the program above was preceded by incubations for RNase or DNase digestion. One fifth to one third of each reaction was run on a 3% NuSieve 3:1 agarose gel (FMC, Rockland ME) in TBE buffer with ethidium bromide, and bands were visualized by UV illumination. Southern blotting was done using the PosiBlot apparatus (Stratagene, La Jolla CA) according to manufacturer's instructions. Random primed probes were made using Stratagene's Random PrimeIt II procedure. Hybridization of the labeled probe to the membrane was done using Amersham Rapid-Hyb solution, followed by high stringency washes at 65° C in 0.1% SSC and 0.1% SDS. The template used to generate the aggrecan probe was a plasmid that has sequence coding for the G3 domain of human aggrecan. The template used to generate the GAPDH probe is a plasmid that has sequence coding for human GAPDH.

EXAMPLE 2

Following the general procedure, set forth above, an assay was performed on amounts of purified total RNA as indicated in Figures 2a and 2b from chondrocytes (top) and fibroblasts (bottom). The aggrecan band is 280 bp, and the GAPDH band is 146 bp. The bottom band is approximately 70 bp and is a result of primer-primer interactions. Figure 2a shows an ethidium bromide stained agarose gel, and Figure 2b shows a Southern blot of the gels hybridized with human aggrecan and GAPDH probes.

EXAMPLE 3

20 ng of purified total RNA from chondrocytes and fibroblasts was digested for 135 minutes at 45° C with or without RNase A/T₁ (20U/800U per ml) in 1x RT-PCR buffer without rTth or primers. After RNase digestion, aggrecan and GAPDH primers and rTth enzyme were added, and RT-PCR thermocycler program was started. Bands corresponding to amplified aggrecan (280 bp), GAPDH (146 bp), and primer-primer interactions (approx. 70 bp) are visible on an ethidium bromide stained agarose gel shown in Figure 4.

EXAMPLE 4

100 ng purified total RNA from chondrocytes and fibroblasts was digested in 1x RT-PCR buffer without rTth or primers for 50 minutes at 37° C with no enzyme (lanes 1 and 2), RNase A/T₁ (20U/800U per ml) (lanes 3 and 4), DNase I (80U/ml) (lanes 5 and 6), or both (lanes 7 and 8). After digestion, the reactions were heated to 90° C of two minutes, aggrecan and GAPDH primers and rTth enzyme were added, and the thermocycler program was started. Bands corresponding to amplified aggrecan (280 bp), GAPDH (146 bp), and primer-primer interactions (approx. 70 bp) are visible on an ethidium bromide stained agarose gel as shown in Figure 5.

EXAMPLE 5

400 ng purified total RNA from chondrocytes and fibroblasts was digested in 1x RT-PCR buffer without rTth or primers for 60 minutes at 37° C with RNase A/T₁ (20U/800U per ml). After digestion, aggrecan and GAPDH primers were added, the reaction was heated to 90° C, rTth enzyme was added, and the thermocycler program was started. A sample of the reaction was run on an agarose gel, blotted to a charged nylon membrane, and hybridized

with a human GAPDH probe. Bands corresponding to amplified genomic DNA (250 bp) and cDNA (146 bp) are visible in Figure 6.

EXAMPLE 6

20,000 intact chondrocytes and fibroblasts that had been pelleted in a PCR tube and resuspended in 1x RT-PCR buffer without rTth or primers were digested for 5 minutes at 37° C with RNase A/T₁ (20U/800U per ml), or were given 40U/ml RNase inhibitor. Separate reactions were performed containing only aggrecan primers or only GAPDH primers. The reaction was heated to 90° C, rTth enzyme was added, and the thermocycler program was started. Bands corresponding to aggrecan (280 bp), GAPDH (146 bp), and primer-primer interactions (approx. 70 bp) are visible on the ethidium bromide stained agarose gel as shown in Figure 7.

EXAMPLE 7

Serial dilutions of intact chondrocytes and fibroblasts in PBS were pelleted to the bottom of a PCR tube, and the PBS supernatant was removed. The reaction mixture including aggrecan and GAPDH primers and rTth enzyme, preheated to 90° C, was added to the cell pellet and the thermocycler program was started. Bands corresponding to aggrecan (280 bp), GAPDH (146 bp), and primer-primer interactions (approx. 70 bp) are visible on the ethidium bromide stained agarose gel in Figure 8.

EXAMPLE 8

An assay was performed on serial dilutions of intact chondrocytes and fibroblasts as in Example 7. A sample of the reaction was run on an agarose gel, blotted to charged nylon membrane, and hybridized with human aggrecan and GAPDH probes. Bands corresponding to

aggrecan (280 bp), GAPDH (146 bp) and primer-primer interactions (approx. 70 bp) are visible in Figure 9.

EXAMPLE 9

10,000 intact chondrocytes were pelleted in a PCR tube and resuspended in 1x RT-PCR buffer (including aggrecan and GAPDH primers) with RNase A/T₁ (20U/800U per ml), or 40U/ml RNase inhibitor. Samples were either immediately put at 90° C, or were subjected to a 5 minute digestion at 37° C before being heated to 90° C. Enzyme rTth was added, and the thermocycler program was started. Bands corresponding to aggrecan (280 bp) and GAPDH (146 bp) are visible on the ethidium bromide stained agarose gel in Figure 10.

WHAT IS CLAIMED IS:

1. A method for determining whether target cells are present in a population of cells comprising:

a) identifying a marker for the target cells and generating primers specific for the mRNA encoding said marker,

b) forming a reaction mixture comprising RT-PCR reaction medium, a cell lysing agent, the primers generated in step (a), and the target cells, and maintaining the reaction mixture at a sufficient temperature to prevent degradation of the mRNA,

c) amplifying said mRNA with said primers using the RT-PCR procedure to generate specific amplified DNA, and

d) detecting the presence or absence of said specific amplified DNA as an indication of the presence or absence of the target cells in the cell population.

2. The method of Claim 1 wherein the cell population contains more than one type of cell.

3. The method of Claim 1 wherein the temperature in step (b) is maintained in the range from about 60° C to about 90° C.

4. The method of Claim 1 wherein the target cells are selected from the group consisting of chondrocyte cells, keratinocyte cells, cells containing viral RNA, cells containing bacterial RNA, cells expressing an mRNA isoform with a deletion or insertion mutation, and RNA transcribed in cancer.

5. The method of Claim 4 wherein the target cells are chondrocyte cells or keratinocyte cells.

6. The method of Claim 5 wherein the target cells are chondrocyte cells.

7. The method of Claim 1 wherein the marker is aggrecan or keratin.
8. The method of Claim 7 wherein the marker is aggrecan.
9. The method of Claim 8 wherein the primer is SEQ ID NO:1 or SEQ ID NO:2.
10. The method of Claim 1 wherein the target cells are obtained from a biopsy performed on a human patient.
11. The method of Claim 10 wherein the biopsy contains human cartilage tissue.
12. The method of Claim 11 wherein the target cells are human chondrocyte cells.
13. The method of Claim 1 wherein there are fewer than about 1,000 target cells in the cell population.
14. The method of Claim 13 wherein there are fewer than about 100 target cells in the cell population.
15. The method of Claim 1 wherein the reaction mixture also includes an RNase inhibitor.

1/11

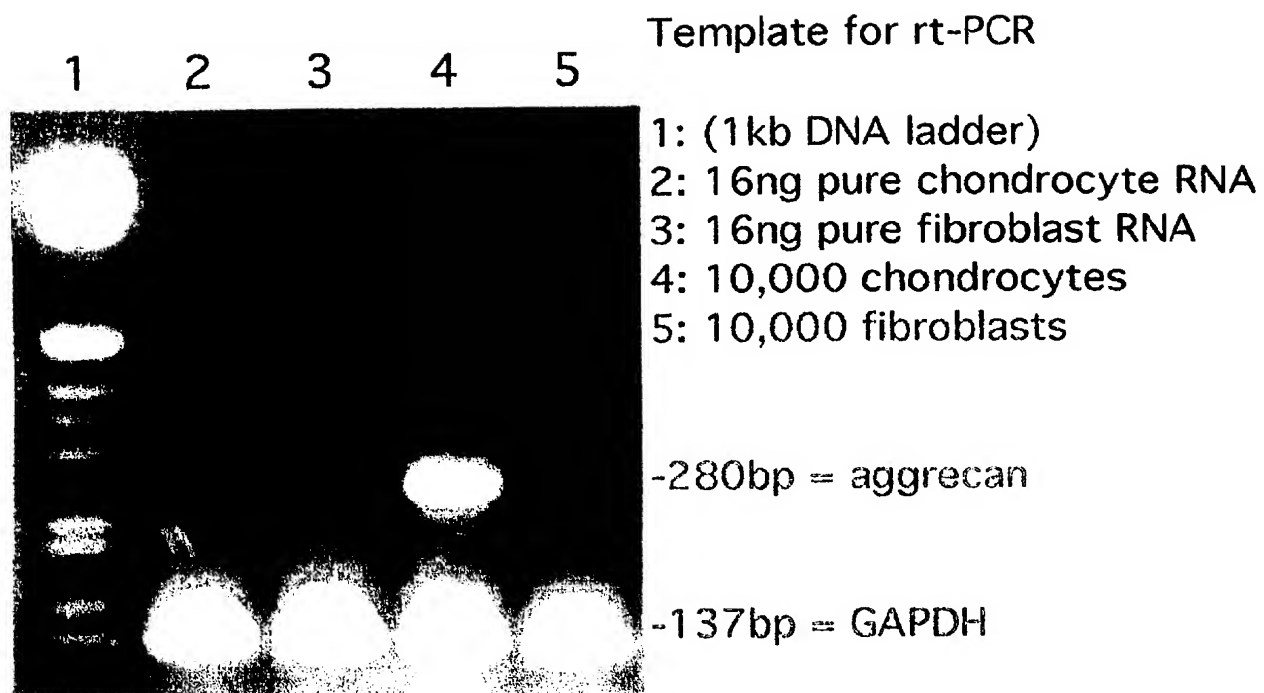


FIG. 1

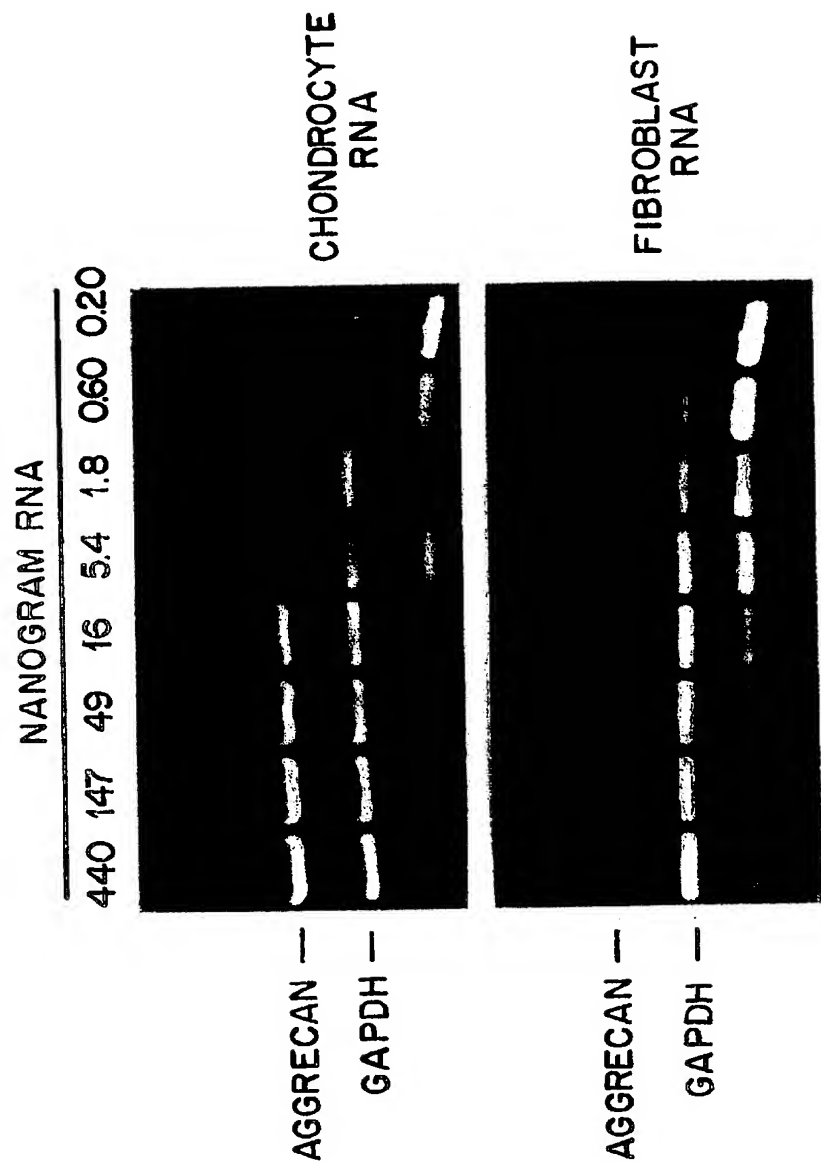


FIG. 2A

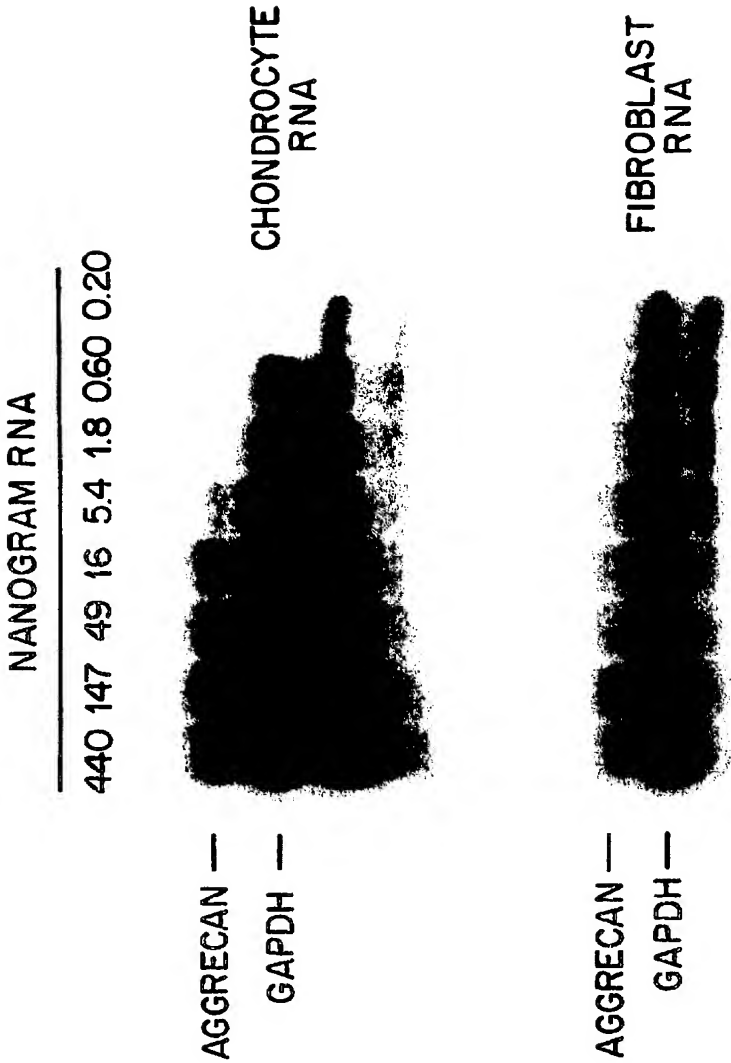


FIG. 2B

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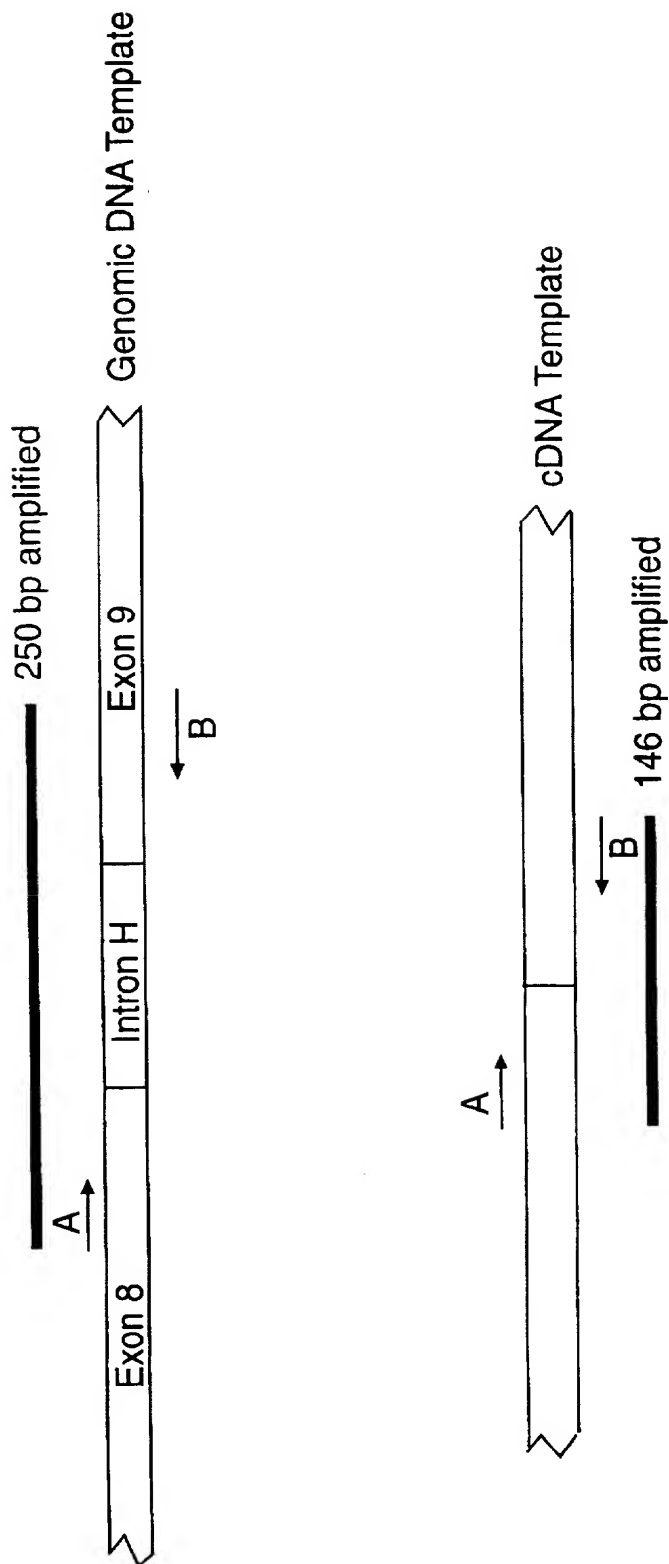


FIG. 3

5/11

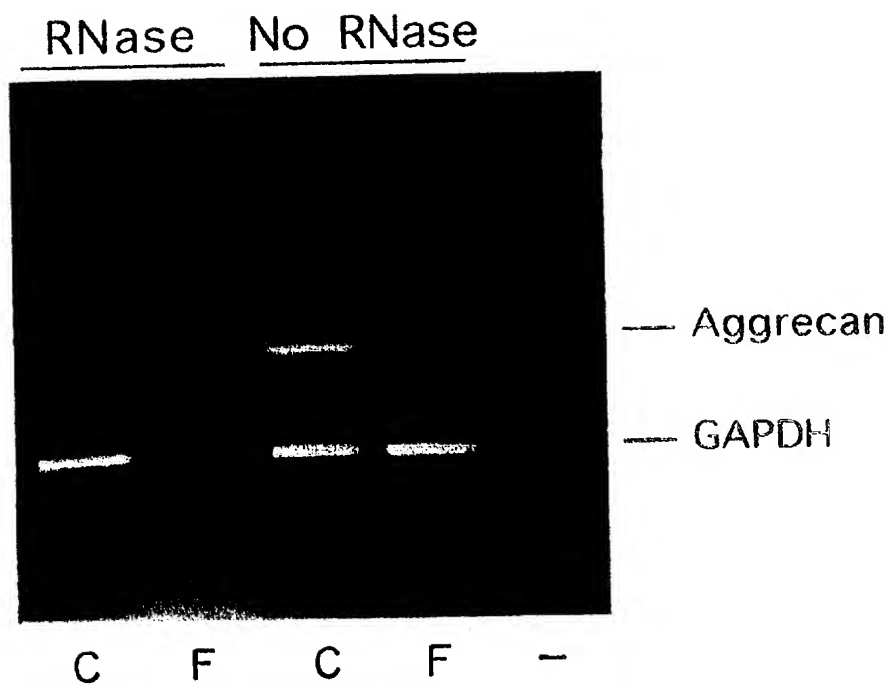


FIG. 4

6/11

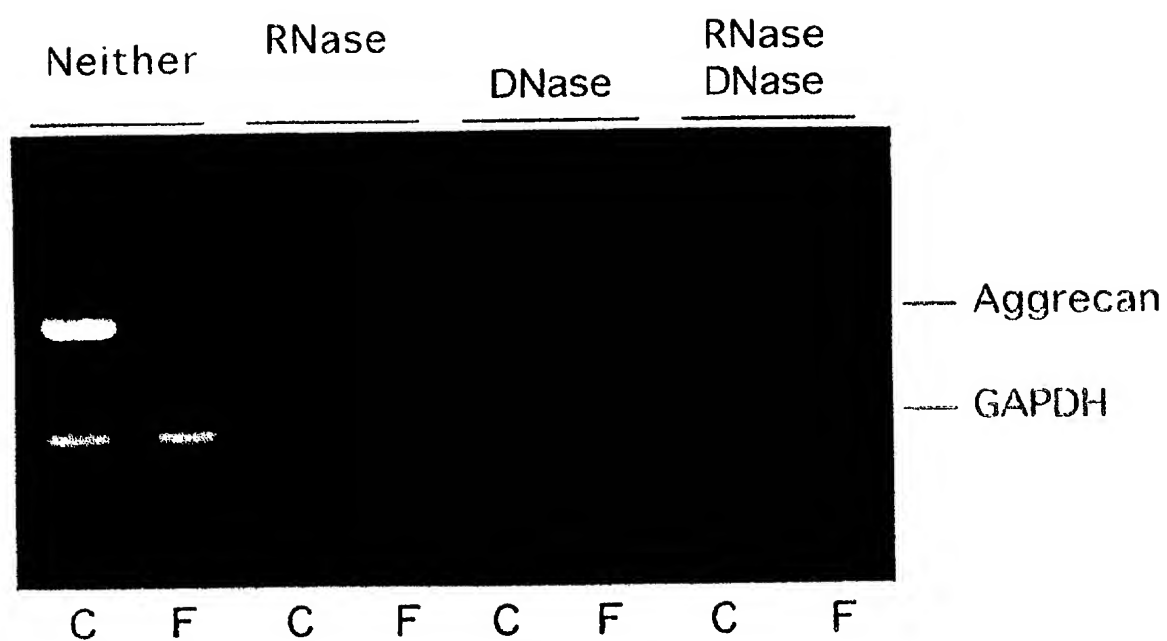


FIG. 5

7/11

CHONDROCYTE

FIBROBLAST

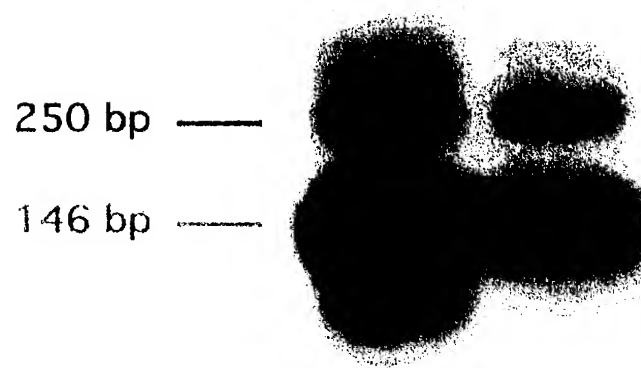


FIG. 6

8/11

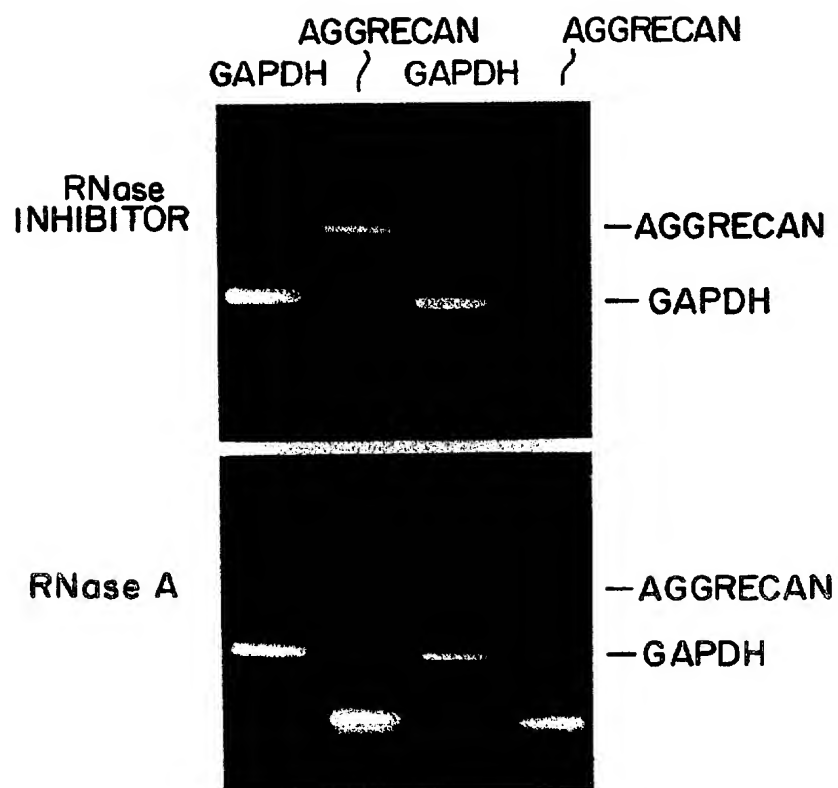


FIG. 7

9/11

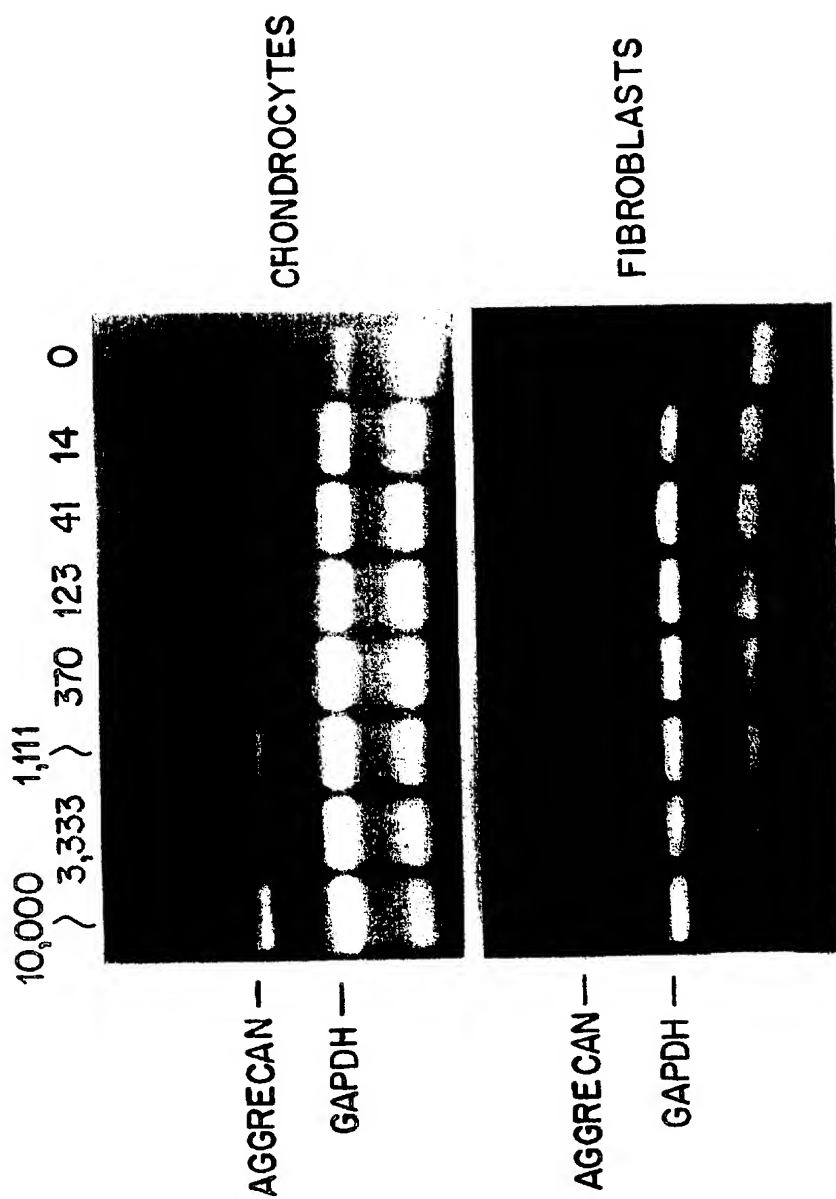


FIG. 8

10/11



FIG. 9

11/11

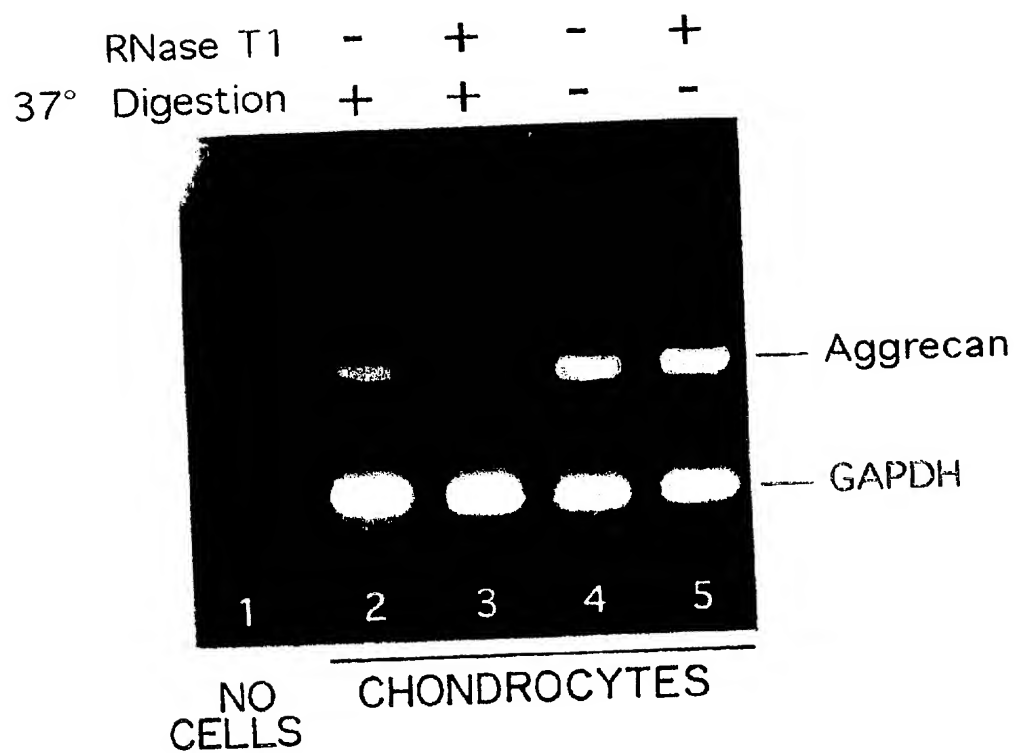


FIG. 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/13131**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : C12Q 1/68, 1/70; C12P 19/34; C12N 9/00; C07H 21/02, 21/04

US CL : 435/6, 5, 91.2, 174; 536/23.1, 24.3, 24.32, 24.33

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 5, 91.2, 174; 536/23.1, 24.3, 24.32, 24.33

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,E	US 5,656,462 A (KELLER et al) 12 August 1997, column 12 lines 17-46, column 18, lines 51-65	1-15
Y, P	US 5,643,730 A (BANKER et al) 01 July 1997, see entire document.	1-15
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☐ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* "A"	document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

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INTERNATIONAL SEARCH REPORT

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B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, BIOSIS, BIOTECHABS, BIOTECHDS, CAPLUS, CANNCERLIT, AIDSLINE, DRUGU, EMBASE, EUROPATFULL, EUROPEX, MEDLINE, SCISEARCH, DISSABS, TOXLINE, TOXLIT, WPIDS, INPADOC, JAPIO, USPATFULL

search terms: RT PCR, RNA PCR, reverse transcriptase, PCR, primers, lysing, lysed, lysis, cells, pellet, chondrocyte, keratinocytes, cancer, tumors, virus, viral, bacterial, pathogens, deletion, insertion, ,aggreacan, keratin, biopsies or biopsy, tissue sections, cartilage, RNase inhibitors, DEPC, VRC, vanadyl